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UTILITY PATENT APPLICATION TRANSMITTAL
(Large Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
BMID9975US

Total Pages in this Submission
64

JC913 U.S. PTO 09/28/00

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TO THE ASSISTANT COMMISSIONER FOR PATENTS
Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

PROCESS FOR THE RECOMBINANT PRODUCTION OF HOLO-CITRATE LYASE

and invented by:

Michael BOTT, Peter DIMROTH and Karin SCHNEIDER

If a CONTINUATION APPLICATION, *check appropriate box and supply the requisite information:*

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

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Which is a:

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Enclosed are:

Application Elements

1. ☐ Filing fee as calculated and transmitted as described below

2. ☒ Specification having 27 pages and including the following:

   a. ☒ Descriptive Title of the Invention

   b. ☐ Cross References to Related Applications *(if applicable)*

   c. ☐ Statement Regarding Federally-sponsored Research/Development *(if applicable)*

   d. ☐ Reference to Microfiche Appendix *(if applicable)*

   e. ☒ Background of the Invention

   f. ☒ Brief Summary of the Invention

   g. ☒ Brief Description of the Drawings *(if drawings filed)*

   h. ☒ Detailed Description

   i. ☒ Claim(s) as Classified Below

   j. ☒ Abstract of the Disclosure

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Application Elements (Continued)

3. ☒ Drawing(s) *(when necessary as prescribed by 35 USC 113)*
   a. ☐ Formal Number of Sheets _____
   b. ☒ Informal Number of Sheets **2**
4. ☒ Oath or Declaration
   a. ☐ Newly executed *(original or copy)* ☒ Unexecuted
   b. ☐ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*
   c. ☒ With Power of Attorney ☐ Without Power of Attorney
   d. ☐ *DELETION OF INVENTOR(S)*
   Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference *(usable if Box 4b is checked)*
   The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Computer Program in Microfiche *(Appendix)*
7. ☐ Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all must be included)*
   a. ☐ Paper Copy
   b. ☐ Computer Readable Copy *(identical to computer copy)*
   c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☐ Assignment Papers *(cover sheet & document(s))*
9. ☐ 37 CFR 3.73(B) Statement *(when there is an assignee)*
10. ☐ English Translation Document *(if applicable)*
11. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations
12. ☒ Preliminary Amendment (to follow)
13. ☒ Acknowledgment postcard
14. ☒ Certificate of Mailing
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Accompanying Application Parts (Continued)

15. ☒ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. ☒ Additional Enclosures *(please identify below):*

General Appointment of Representative for U.S. Patent and Trademark Office Matters.

Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra		Rate	Fee
Total Claims		- 20 =	0	x	$18.00	$0.00
Indep. Claims		- 3 =	0	x	$78.00	$0.00
Multiple Dependent Claims (check if applicable) ☐						$0.00
					BASIC FEE	$0.00
OTHER FEE *(specify purpose)* _____						$0.00
					TOTAL FILING FEE	$0.00

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Signature

Dated: **September 28, 2000**

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CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10) Applicant(s): **Michael BOTT, et al.**			Docket No. **BMID9975US**
Serial No. **To Be Assigned**	Filing Date **September 28, 2000**	Examiner **To Be Assigned**	Group Art Unit **To Be Assigned**

Invention: **PROCESS FOR THE RECOMBINANT PRODUCTION OF HOLO-CITRATE LYASE**

I hereby certify that this **Utility Patent Application**
(Identify type of correspondence)

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231 on **September 28, 2000.**
(Date)

Rose Edwards
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Roche Diagnostics GmbH 5234/00/EP

Process for the recombinant production of holo-citrate lyase

The enzyme citrate lyase (EC4.1.3.6) is regarded as a key enzyme of anaerobic citrate degradation and can accordingly be isolated from a number of different prokaryotic cells. The enzyme catalyses the cleavage of citrate into acetate and oxaloacetate. Furthermore it is known that the enzyme complex of the citrate lyase enzyme that has been best examined to date from Klebsiella pneumoniae (formally: Klebsiella aerogenes) is composed of six copies of each of three different subunits and namely an α, β and γ subunit, of a molecular weight of about 550,000 Dalton. In addition it is known that the catalytically active centre is located in the α and β subunit, whereas the γ subunit has the binding site for the prosthetic group 2'-(5"phosphoribosyl)-3'-dephospho CoA. This prosthetic group is bound to the serine residue 14 via a phosphodiester bond.

The citrate lyase enzyme is required in high purity for most applications which are primarily for clinical chemistry and food analysis. Hence the aim is to over-produce the enzyme in an active form in certain host cells by recombinant methods and to isolate it from these cells. Such a process has not yet been described or made known in other ways. Hence citrate lyase is nowadays usually isolated from Klebsiella pneumoniae cells which had been cultured under anaerobic conditions using citrate as the only carbon and energy source. The citrate lyase genes from Klebsiella pneumoniae have been

cloned and sequenced (M. Bott and P. Dimroth, Mol. Microbiol. Vol. 14, 347-356 (1994)). These genes are part of the citC operon which is composed of the five genes citCDEFG. The citC gene codes for citrate lyase ligase which catalyses the formation of an acetyl thioester. The genes citD, citE and citF code for the gamma, beta and alpha subunit of citrate lyase. The protein coded by citG is involved in the biosynthesis of the prosthetic group. Furthermore it is known that the citC operon is induced in the absence of oxygen and in the presence of citrate and Na^+ ions; moreover the expression is strongly dependent on the citA/citB regulation system (M. Bott et al., Mol. Microbiol. Vol. 18, 533-546 (1995); M. Meyer et al., J. Mol. Biol. Vol. 269, 719-731 (1997)).

Expression of the genes coding for citrate lyase from Klebsiella pneumoniae which would preferably be carried out in prokaryotic cells such as E. coli for practical reasons, results in an inactive but nevertheless soluble form of the enzyme (M. Bott and P. Dimroth, Mol. Microbiol. Vol. 14, 347-356 (1994)). The recombinant apo-citrate lyase enzyme can be activated to form the holo-enzyme by subsequent addition of acetyl coenzyme A which is known as a substituent for the acetyl thioester of the native prosthetic group 2'-(5"-phosphoribosyl)-3'-dephospho CoA. However, such an additional activation measure is complicated and laborious. Moreover the necessity to add acetyl CoA is unsuitable for the commercial distribution of citrate lyase or the apo form since the substance decomposes when stored for long periods at 4°C.

Hence the object of the invention is to provide a recombinant, soluble and at the same time active holo-

citrate lyase which eliminates the disadvantages of the known methods.

The object is achieved by a process for the production of a protein with citrate lyase activity by expressing a suitable plasmid in a host organisms whereby the plasmid contains the information of a gene cluster composed of at least six genes and an inducible promoter. The genes comprising the gene cluster code for certain subunits of the protein with citrate lyase activity and/or for a component which participates in the biosynthesis of the complete enzyme. In particular a suitable plasmid contains the genes citC, citD, citE, citF, citG and a DNA fragment that can for example be obtained from E. coli which is located between the genes citF and citG on the E. coli citrate lyase gene cluster. The genes citD, citE and citF code for the corresponding γ, β and α subunits of the enzyme and have molecular weights of about 11,000 Dalton, 32,000 Dalton and 55,000 Dalton. According to the invention it is preferred that one of the genes represents a DNA fragment which codes for a protein containing the motif G(A)-R-L-X-D-L(I)-D-V. A corresponding DNA fragment is particularly preferred which codes for a protein with a molecular weight of about 20,000 Dalton.

In addition it has proven to be advantageous when one gene and optionally a further gene fused to the first gene of the genes comprising the gene cluster is derived from a different organism than the other genes. In particular it has proven to be advantageous when the DNA fragment citX or genes homologous to citX located between citF and citG on the E. coli citrate lyase gene cluster are derived from E. coli, Klebsiella pneumoniae, Haemophilus influenzae or Leuconostoc mesenteroides and

when one or several of the other genes are derived from the microorganism that is specific for the isolated protein having citrate lyase activity which is for example Klebsiella pneumoniae. In Haemophilus influenza, Leuconostoc mesenteroides (S. Bekal et al., J. Bacteriol. Vol. 180, 647-654 (1998)) and Leuconostoc paramesenteroides (M. Martin et al., FEMS Microbiol. Lett. Vol. 174, 231-238 (1999)) the genes citX and citG occur in a fused form. Thus corresponding fusion genes contain the information of two genes. The resulting proteins have a molecular weight of about 52,000 Dalton, have the activities of E. coli CitX and CitG and are thus bifunctional. In the absence of the citX gene or of a gene homologous to citG or of a corresponding citX fusion gene, only the low-molecular apo form (MW 12,000 Dalton, SDS-PAGE) but not the holo form of citrate lyase (MW 14,500 Dalton, SDS-PAGE) could be detected after expression.

According to the invention prokaryotes as well as eukaryotes have proven to be suitable as the host organism. The fact that a soluble active citrate lyase can now be produced in prokaryotes such as e.g. E. coli in a simple manner and in adequate yields without additional activation measures is a major advantage.

Hence it was possible to show that by cloning the entire E. coli citCDEFXG gene cluster under the control of an inducible promoter such as e.g. the lac, lac UV5, T5, tac or T7 promoter, an active enzyme can be expressed having citrate lyase activity even under non-oxygen limiting conditions. Cell extracts containing appropriate expression plasmids result in citrate lyase activities of about 4 to 5 U/mg protein in the cell-free extract whereas cells without recombinant citrate lyase

have no citrate lyase activity when grown aerobically.

In addition the invention concerns the simultaneous expression of the citCDEFG gene cluster from Klebsiella pneumoniae and of the citX gene obtainable from E. coli by which means it is possible to obtain a corresponding citrate lyase in an active form even in prokaryotes and in particular in E. coli.

By this means it was possible to achieve an activity of about 8 U/mg total protein in a cell-free extract under aerobic growth conditions.

The holo-enzyme is purified by methods known to a person skilled in the art. About 100 to 120 µg soluble protein with citrate lyase activity can be obtained from about 1 g of cells (wet weight) using the process according to the invention. The protein determination was carried out according to P.K. Smith et al., Anal. Biochem. Vol. 150, 76-85 (1985) using ovalbumin as a standard. The specific activity of the citrate lyase is ca. 70 U/ml protein (M. Single and P.A. Srere, J. Biol. Chem. Vol. 251 (10), 2911-2615 (1976)). The activity of the holo-enzyme that can be obtained by the process according to the invention is thus ca. 0.5 to 3-fold higher than the activity that was achieved with acetyl CoA and apo-citrate lyase.

Hence the process according to the invention provides for the first time a recombinant protein with improved citrate lyase activity that is both soluble and active.

Furthermore the invention concerns a test kit for the determination of citric acid which is composed

essentially of the following components: a protein obtainable by the process according to the invention with citrate lyase activity, at least one protein with hydrogen-transferring activity, nicotinamide-adenine dinucleotide or an appropriate derivative in a reduced form and optionally suitable stabilizers, activators and/or substances to avoid or reduce interferences i.e. components or reactions which mask or interfere with the actual reaction as well as suitable buffer solutions. In particular L-malate dehydrogenase and L-lactate dehydrogenase come into consideration as proteins with hydrogen-transferring activity. Those substances, additives or measures which help to avoid or at least to delay the degradation of a property or activity that is important for the determination are in principle suitable as stabilizers. Especially when only small amounts of sample material are available or if the samples are very dilute it can be advantageous to add activators.

An additional subject matter of the invention is the use of the recombinant soluble protein with citrate lyase activity to determine citric acid in clinical chemistry, food analysis and as a purity test for cosmetics. In clinical chemistry a corresponding enzymatic test is used primarily to examine fertility and for therapeutic monitoring of patients with kidney stones. In food analysis the most important application is analysis of wines and fruit juices.

The enzymatic method is based on the cleavage of citrate by the enzyme citrate lyase in the presence of Mg^{2+} ions to form oxaloacetate and acetate. In the presence of hydrogen-transferring enzymes such as L-malate dehydrogenase and L-lactate dehydrogenase, oxaloacetate

and its decarboxylation product pyruvate are reduced by reduced NADH or NADPH to form L-malate and L-lactate. The amount of NADH or NADPH is proportional to the amount of citrate and is measured at 334 nm, 340 nm or 365 nm.

Hence the invention also concerns a corresponding test kit for the determination of citric acid which, apart from suitable buffer solutions, contains a recombinant protein with citrate lyase activity, one or several hydrogen-transferring enzymes and a nicotinamide adenine dinucleotide or a corresponding derivative in a reduced form and optionally suitable stabilizers such as thiol reagents.

Figure legends

Figure 1:
A: Function of the various subunits in a reaction catalysed by citrate lyase and activation of the enzyme by citrate lyase ligase. HS-R denotes a prosthetic group.

B. Structure of the prosthetic group of citrate lyase 2'-(5"-phosphoribosyl)-3'-phospho-CoA.

Figure 2:
Citrate lyase gene cluster from Klebsiella pneumoniae (K.p.), Escherichia coli (E.c.) Haemophilus influenzae (H.i.) and Leuconostoc mesenteroides (L.m.). Gene sequences that are homologous to E. coli citX are shown by the light grey shading.

INFORMATION FOR SEQ ID NO. 1:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 36 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

5´- CCCTCTAGAGAACAACATTCGTTGCAAATCGATAAC - 3´

INFORMATION FOR SEQ ID NO. 2:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 38 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

5´- CCGCGAATTCTTAGTTCCACATGGCGAGAATCGGCCAG -3´

INFORMATION FOR SEQ ID NO. 3:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 5484 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

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   1  GAACAACATT CGTTGCAAAT CGATAACAAC ATGCACCTTC AGGATACTAT
         ┌Start citC
  51  TTATTATGTT CGGCAATGAT ATTTTCACCC GCGTAAAACG TTCAGAAAAT
 101  AAAAAAATGG CGGAAATCGC CCAATTCCTG CATGAAAATG ATTTGAGCGT
 151  TGACACCACA GTCGAAGTAT TTATTACCGT AACCCGCGAT GAAAAGCTTA
 201  TCGCGTGCGG TGGAATTGCC GGAAATATTA TTAAATGCGT TGCTATCAGT
 251  GAATCCGTCC GCGGTGAAGG ACTGGCGCTG ACATTAGCCA CTGAATTGAT
 301  AAACCTCGCC TATGAGCGGC ACAGCACGCA TCTGTTTATT TATACCAAAA
 351  CCGAATACGA GGCGCTGTTC CGCCAGTGCG GTTTTTCCAC GCTGACCAGC
 401  GTACCCGGCG TGATGGTGCT GATGGAAAAC AGCGCCACGC GACTGAAACG
 451  CTATGCCGAA TCGCTGAAAA AATTTCGTCA TCCAGGGAAC AAGATTGGCT
 501  GCATTGTGAT GAACGCCAAT CCCTTTACGA ATGGTCACCG TTATCTGATT
 551  CAACAGGCTG CGGCACAGTG CGACTGGTTG CATCTGTTTT TAGTCAAAGA
 601  AGATTCTTCA CGCTTCCCCT ATGAAGACCG GCTGGATTTG GTGTTAAAAG
 651  GCACCGCCGA TATTCCACGC CTGACTGTGC ATCGTGGCTC CGAATACATC
 701  ATCTCCCGCG CTACGTTCCC TTGCTACTTC ATTAAAGAAC AGAGCGTCAT
 751  TAACCATTGT TACACCGAAA TTGATCTGAA GATTTTCCGT CAGTACCTCG
 801  CTCCCGCGCT GGGTGTAACT CACCGCTTTG TCGGTACTGA ACCCTTTTGT
 851  CGCGTTACCG CCCAGTACAA CCAGGATATG CGCTACTGGC TGGAAACGCC
 901  GACTATCTCC GCACCGCCCA TCGAACTGGT TGAAATTGAG CGGCTGCGTT
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 951  ACCAGGAGAT GCCGATATCC GCTTCCCGGG TACGTCAACT GCTGGCGAAA
1001  AACGATCTCA CGGCTATCGC GCCGCTGGTC CCTGCAGTCA CGCTGCATTA
1051  TTTGCAGAAC CTGCTTGAGC ACTCCCGCCA GGACGCGGCA GCTCGTCAAA
          Stop citC┐                        ┌Start citD
1101  AGACCCCCGC ATGAGAAACA GGTGAAAAAT GAAAATAAAC CAGCCCGCCG
1151  TTGCAGGCAC CCTTGAGTCT GGGGATGTGA TGATACGCAT CGCCCCACTC
1201  GATACGCAGG ATATCGACCT GCAAATCAAT AGCAGCGTTG AGAAACAGTT
1251  TGGCGATGCA ATTCGCACCA CCATTCTGGA CGTTCTCGCC CGCTACAACG
1301  TGCGCGGCGT ACAGCTGAAT GTCGATGACA AAGGCGCACT GGACTGCATT
1351  TTACGTGCAC GACTGGAAGC CCTGCTGGCA CGCGCCAGCG GTATCCCGGC
                    Stop citD┐
1401  TCTGCCATGG GAGGATTGCC AATGATTTCC GCTTCGCTGC AACAACGTAA
                              └Start citE
1451  AACTCGCACC CGCCGCAGCA TGTTGTTTGT GCCTGGTGCC AATGCCGCGA
1501  TGGTCAGCAA CTCCTTCATC TACCCGGCTG ATGCCCTGAT GTTTGACCTC
1551  GAAGACTCCG TAGCATTGCG TGAAAAAGAC ACCGCCCGCC GCATGGTTTA
1601  CCACGCGCTG CAACATCCGC TGTATCGCGA TATTGAAACC ATTGTGCGTG
1651  TCAACGCGCT GGATTCCGAA TGGGGTGTTA ACGACCTGGA AGCCGTCGTT
1701  CGCGGTGGTG CGGACGTTGT GCGTCTGCCG AAAACCGATA CCGCTCAGGA
1751  TGTTCTGGAT ATTGAAAAAG AGATCCTGCG TATCGAAAAA GCCTGTGGTC
1801  GTGAACCCGG CAGCACCGGC CTGCTGGCGG CGATTGAATC TCCGCTGGGG
1851  ATTACCCGCG CAGTGGAAAT CGCTCACGCT TCCGAGCGTT TGATCGGTAT
1901  CGCCCTCGGT GCAGAAGACT ATGTGCGCAA CCTGCGTACA GAACGCTCCC
1951  CGGAAGGAAC TGAACTGCTG TTCGCACGCT GTTCCATTTT GCAGGCCGCG
2001  CGCTCTGCGG GTATTCAGGC GTTCGATACC GTCTATTCCG ACGCTAACAA
2051  CGAAGCCGGA TTTCTGCAAG AAGCCGCCCA CATCAAACAG CTGGGCTTTG
2101  ACGGCAAATC GCTGATCAAC CCGCGTCAGA TTGATCTGCT GCACAACCTC
2151  TACGCACCGA CCCAGAAAGA AGTGGATCAC GCCCGCCGCG TCGTAGAAGC
2201  CGCTGAAGCC GCCGCTCGCG AAGGCCTCGG CGTGGTTTCC CTGAACGGCA
2251  AGATGGTGGA CGGTCCGGTT ATCGATCGCG CCCGTCTGGT GCTCTCCCGT
                          Stop citE┐              ┌Start citF
2301  GCAGAACTTT CCGGCATCCG CGAAGAATAA GGCAATCAAA ATGACGCAGA
2351  AAATTGAACA ATCTCAACGA CAAGAACGGG TAGCGGCCTG GAATCGTCGC
2401  GCTGAATGCG ATCTTGCCGC TTTCCAGAAC TCGCCAAAGC AAACCTACCA
2451  GGCTGAAAAA GCGCGCGATC GCAAACTGTG CGCCAACCTG GAAGAAGCGA
2501  TTCGTCGCTC TGGTTTACAG GACGGCATGA CGGTTTCCTT CCATCACGCT
2551  TTCCGTGGCG GTGACCTGAC CGTCAATATG GTGATGGACG TCATCGCGAA
2601  GATGGGCTTT AAAAACCTGA CCCTGGCGTC CAGCTCCCTG AGTGATTGCC
2651  ATGCGCCGCT GGTAGAACAC ATTCGCCAGG GCGTGGTTAC CCGCATTTAT
2701  ACCTCCGGCC TGCGTGGTCC ACTGGCGGAA GAGATCTCCC GTGGTCTGCT
2751  GGCAGAACCG GTGCAGATCC ACTCTCACGG CGGTCGTGTG CATCTGGTAC
2801  AGAGCGGCGA ACTGAATATC GACGTGGCTT TCCTCGGCGT CCCGTCCTGT
2851  GATGAATTCG GTAATGCCAA CGGCTACACC GGTAAAGCCT GCTGCGGCTC
2901  CCTCGGCTAT GCAATAGTTG ATGCCGACAA CGCAAAACAG GTCGTGATGC
2951  TTACCGAAGA ACTGCTGCCT TATCCGCATA ATCCGGCAAG CATTGAGCAA
3001  GATCAGGTTG ATTTGATCGT CAAAGTTGAC CGCGTTGGCG ATGCTGCAAA
3051  AATCGGCGCT GGCGCGACCC GTATGACCAC TAACCCGCGC GAACTGCTTA
3101  TTGCCCGTAG CGCTGCGGAT GTGATTGTCA ACTCTGGCTA CTTCAAAGAA
3151  GGTTTCTCCA TGCAAACCGG CACCGGCGGC GCATCGCTGG CGGTAACCCG
3201  TTTCCTGGAA GACAAAATGC GTAGCCGCGA TATTCGCGCC GACTTCGCCC
3251  TTGGCGGTAT TACCGCGACG ATGGTTGACC TGCACGAAAA AGGTCTGATC
3301  CGCAAACTGC TGGATGTGCA GAGCTTTGAC AGCCATGCTG CGCAATCGCT
3351  GGCCCGTAAC CCCAATCACA TCGAAATCAG CGCCAACCAG TACGCTAACT
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3451  GCGCTGGAAA TTGACACCCA GTTCAACGTT AACGTGCTGA CCGGCTCTGA
3501  CGGCGTACTG CGTGGTGCTT CCGGTGGTCA CTGCGATACC GCGATTGCCT
3551  CTGCGCTTTC CATCATCGTC GCGCCGCTGG TACGCGGTCG TATTCCGACT
3601  CTGGTGGATA ACGTACTGAC CTGCATCACC CCAGGCTCCA GTGTCGATAT
3651  TCTGGTCACA GACCACGGTA TCGCAGTTAA CCCGGCACGT CCGGAACTGG
3701  CAGAACGTCT GCAGGAAGCG GGCATTAAAG TGGTTTCCAT TGAGTGGCTG
3751  CGCGAACGTG CGCGTCTGCT GACCGGTGAA CCACAGCCGA TTGAATTCAC
3801  AGACCGCGTC GTTGCCGTTG TGCGTTACCG CGATGGCTCG GTGATCGATG
                  Stop citF┐      ┌Start citX
3851  TTGTGCATCA GGTGAAGGAA TAAGCCATGC ACCTGCTTCC TGAACTCGCC
3901  AGCCACCATG CGGTATCAAT TCCCGAGCTG CTCGTCAGCC GGGATGAAAG
3951  GCAAGCACGG CAACACGTCT GGCTCAAGCG CCATCCTGTT CCACTGGTCT
4001  CCTTTACCGT GGTTGCGCCT GGGCCGATTA AAGACAGCGA GGTCACACGC
4051  CGAATTTTTA ATCATGGCGT GACAGCCTTG CGTGCCTTAG CCGCAAAACA
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4201  ATTGAGCTTG AACATAGTCA TCCTCTCGGG CGGTTATGGG ATATCGATGT
4251  CCTGACGCCC GAAGGCGAAA TTCTCTCCCG CCGCGACTAT TCACTGCCGC
4301  CTCGCCGCTG CCTGTTGTGC GAACAAAGCG CAGCCGTCTG CGCGCGTGGA
4351  AAAACCCATC AACTGACCGA TTTACTCAAC CGCATGGAGG CACTGCTGAA
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4451  TTAATCGATG AGTACGCCCT GCTGGGCTGG CGCGCCATGC TGACTGAAGT
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4601  GGCTGGCTAC CCCGTTTCAT TGAATTTGGT GCCTGTAGTG CGGAAATGGC
4651  ACCAGAAGCG GTACTCCACG GATTACGCCC AATTGGTATG GCTTGCGAAG
4701  GTGATATGTT CCGCGCCACT GCGGGCGTAA ACACGCATAA AGGCAGCATT
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4801  CCAACCGGTA ACGCCAACAA CCGTTTGTTC TACGGCGGCA AGTTTCTGCC
4851  GTGGCCTGAC CGATCGCGAA CTGCGTACCA ATAATTCACA ACTGACGGCA
4901  GGTCAACGGT TGTACCAACA GCTTGGCCTT ACCGGCGCAC GCGGTGAAGC
4951  CGAAGCGGGT TATCCACTGG TGATCAATCA CGCCTTGCCG CATTACCTCA
5001  CTCTGCTGGA TCAGGGGTTA GATCCTGAAC TGGCATTGCT CGATACCTTG
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5101  CGAGGGGGGC CTGCGCTGGC TACAGCGCGA GGCGCAAACA TTATTGCAAA
5151  AAGGGGGCAT TCGAACCCCC GCCGATCTCG ATTATCTCCG GCAGTTCGAC
5201  AGGGAGTGTA TCGAACGAAA TCTCAGTCCA GGCGGCAGTG CTGACCTACT
                              Stop citG┐
5251  GATCCTTACC TGGTTTTTAG CACAGATTTA ATTATTTAAG CACTTGATAA
                                         ┌Start citT
5301  ATTTGGAAAT ATTAATTTTC GGAGAACCCG TATGTCTTTA GCAAAAGATA
5351  ATATATGGAA ACTATTGGCC CCACTGGTGG TGATGGGTGT CATGTTTCTT
5401  ATCCCTGTCC CCGACGGTAT GCCGCCGCAG GCATGGCATT ACTTCGCTGT
5451  GTTTGTGGCA ATGATTGTCG GCATGATCCT CGAG
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INFORMATION FOR SEQ ID NO. 4:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 33 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

5´- AAATTTCATATGCACCTGCTTCCTGAACTCGCC - 3´

INFORMATION FOR SEQ ID NO. 5:

SEQUENCE CHARACTERISTICS:
(A) LENGTH : 36 base pairs
(B) TYPE : nucleic acid
(C) STRANDNESS : single
(D) TOPOLOGY : linear

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INFORMATION FOR SEQ ID NO. 6:

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(C) STRANDNESS : single
(D) TOPOLOGY : linear

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301  GCTCGCGACC TCAAGCTCGC CACCATTGAG CTTGAACATA GTCATCCTCT
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5581 TGAACAAAGA TCT
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The invention is further elucidated by the following examples:

Example 1:

Cell culture

The following strains and plasmids were used: E. coli DH5α or BL21 (DE3) (F.W. Studiar and B.A. Mofatt, J. Mol. Biol. Vol. 189, 113-130 (1986)) and pACYC184 (A.C.Y. Chang et al., J. Bacteriol. Vol. 134, 1141-1156 (1978)). The E. coli cells were routinely cultured in Luria Bertani (LB) medium at 37°C according to J. Sambrook et al., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2nd Edition 1989). Antibiotics were added at the following final concentrations: 200 µg/ml ampicillin, 50 µg/ml chloroamphenicol and 50 µg/ml kanamycin. The E. coli strain DH5α was used as the host organism for the cloning. The E. coli BL21 (DE3) cells which contain the phage T7 polymerase gene under the control of a lacUV5 promoter (F.W.Studier and B.A. Moffatt, supra) served as a host for the expression of the target genes of pT7-7 and pET derivatives. The cultures for the expression were prepared as follows. After centrifugation (3000 g, 8 min) of a preculture of 40 ml which had been incubated overnight at 37°C, the cells were resuspended in 20 ml fresh LB medium. The cell suspension was subsequently

used to inoculate 2 L of the same medium which contained appropriate antibiotics and the culture was incubated at 37°C in a shaker (180 rpm). When the OD_{600} reached a value between 0.5 and 0.8, the expression of the target genes was induced by adding IPTG (isopropyl-β-D-thiogalactoside) at a final concentration of 1 mM and the culture was incubated for a further 3 hours at 37°C in a shaker (180 rpm). Subsequently the cells were harvested by centrifugation (30 min at 3000 g), washed once with 20 ml 50 mM potassium phosphate, pH 7.0, 1 mM $MgCl_2$ and stored at -20°C.

Example 2:

Isolation of the genes and gene cluster

For the construction of the expression plasmid which contains the E. coli citCDEFXG gene cluster, a 6.9 kb fragment from the chromosomal DNA of E. coli was amplified by means of PCR with the primers eccl-for (SEQ ID NO.1) and ec-citT-rev (SEQ ID NO.2) using the Expand High Fidelity PCR System from Roche Diagnostics. The 6.9 kb PCR fragment which additionally contains the citT gene (K.M. Pos et al., J. Bacteriol. Vol. 180, 4160-4165 (1998)), was cleaved with the restriction endonucleases XbaI and XhoI and the resulting 5.5 kb fragment (SEQ ID NO.3) and an expression vector that was also linearized correspondingly such as pKK177-3Hb, pKKT5, pUC18, pT7, pET24b were separated on an agarose gel and the appropriate bands were isolated (QIAEX kit from the Diagen Company). Subsequently the PCR fragment and the vector fragment were ligated together using T4 DNA ligase. For this 1 µl (20 ng) vector fragment and 3 µl (100 ng) PCR fragment, 1 µl 10 x ligase buffer (Maniatis et al., 1989 B.27), 1 µl T4 DNA ligase, 4 µl sterile redistilled H_2O were pipetted, carefully mixed

and incubated overnight at 16°C. The insert obtained from the PCR starts 55 bp before the citC start codon and ends 203 bp downstream of the citG stop codon.

For the construction of the expression plasmid which contains the citX gene from E. coli (SEQ ID NO.3), the citX gene was amplified by PCR from the chromosomal DNA with the primers ec-citX-for (SEQ ID NO.4) and ec-citX-rev (SEQ ID NO.5) using the Pfu DNA polymerase (Stratagene). The start codon is part of an NdeI restriction endonuclease cleavage site and a XhoI restriction endonuclease cleavage site is located directly behind the stop codon. After digestion of the PCR product with NdeI and XhoI, the resulting 555 bp DNA fragment (SEQ ID NO.6) was ligated into appropriately linearized expression vectors (as described above).

The construction of the expression plasmid which contains the citCDEFG gene cluster of Klebsiella pneumoniae is described in M. Bott and P. Dimroth, Molecular Microbiology Vol. 14 (2), 347-356 (1994). The sequence of the citCDEFG gene cluster is shown in SEQ ID NO.7.

Example 3:

Transformation of the various expression plasmids in various E. coli expression strains

Competent cells of various E. coli strains were prepared according to the method of Hanahan (J. Mol. Biol. Vol. 166, 557 ff. (1983)). 200 µl of cells prepared in this manner were mixed with 20 ng of the corresponding expression plasmids. After 30 minutes incubation on ice, a heat shock was carried out (90 sec. at 42°C).

Subsequently the cells were transferred to 1 ml LB medium and incubated for 1 hour at 37°C for the phenotypic expression. Aliquots of this transformation mixture were plated on LB plates containing the appropriate antibiotic as a selection marker and incubated for 15 hours at 37°C.

Example 4:

Expression of the various target genes

After centrifugation (3000 g, 8 min) of 40 ml preculture which had been grown overnight at 37°C, the cell pellet was resuspended in 20 ml fresh LB medium. The cell suspension was then used to inoculate 2 l LB medium containing the appropriate antibiotics. This cell culture was incubated at 37°C in a shaker (180 rpm). The expression of the target genes was induced at an optical density (measured at 600 nm) of 0.5 - 0.8 by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG, final concentration) and the cultures were incubated for a further 3 hours at 37°C and 180 rpm. Afterwards the cells were harvested by centrifugation (30 min. at 3000 g), washed once in 20 ml 50 mM potassium phosphate, pH 7.0 and frozen at -20°C.

For the cell extract preparation, 1 g cells (wet weight) were resuspended in 4 ml cold 50 mM potassium phosphate, 1 mM $MgCl_2$ pH 7.0. After adding a protease inhibitor cocktail (Roche Diagnostics) and DNAseI to a final concentration of 25 mg/ml, the cells were lysed by a three-fold passage in a French press at 108 Mpa. Intact cells and cell debris were removed by centrifugation (30 min. at 27,000 g). The cell-free supernatant was separated from the membrane fraction by ultracentrifugation (1 H at 150,000 g) and the resulting

cell extract can then be used directly for enzymatic studies and for protein purification.

Example 5:

Citrate lyase activity test

The citrate lyase activity was measured at 25°C in a spectrophotometric test coupled with malate dehydrogenase from Roche Diagnostics. The test mixture contained in a final volume of 1 ml 50 mM glycylglycine pH 7.9, 5 mM potassium citrate, 2 mM $ZnCl_2$, 0.5 mM NADH, 30 U malate dehydrogenase (Roche Diagnostics) and 10 µl or 20 µl cell extract. The oxidation of NADH was measured in a spectrophotometer at 365 nm (ε = 3.4 mM^{-1} cm^{-1}). One enzyme unit (unit) is defined as 1 µmol citrate which is degraded per minute to acetate and oxaloacetate.

SEQUENCE LISTING

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<130> 523400EP

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Claims

1. Process for the production of a protein with citrate lyase activity by expressing a suitable plasmid in a host organism and isolating the protein in an active form, wherein the plasmid contains the information from a gene cluster composed of at least six genes and an inducible promoter.

2. Process as claimed in claim 1, wherein the genes code for certain subunits of the protein having citrate lyase activity and/or for components that contribute to the biosynthesis of the complete enzyme.

3. Process as claimed in one of the claims 1 or 2, wherein the plasmid contains the genes citC, citD, citE, citF, citG and a DNA fragment obtainable from E. coli that is located between citF and citG on the E. coli citrate lyase gene cluster.

4. Process as claimed in claim 3, wherein the DNA fragment codes for a 20 kDa protein.

5. Process as claimed in claim 3 or 4, wherein the DNA fragment codes for a protein containing the motif G(A)-R-L-X-D-L(I)-D-V.

6. Process as claimed in one of the claims 1 to 5, wherein at least one gene is obtainable from E. coli, Haemophilus influenzae, Klebsiella pneumoniae or Leuconostoc mesenteroides.

7. Process as claimed in one of the claims 1 to 6, wherein at least four genes are derived from the microorganism that is specific for the isolated protein with citrate lyase activity.

8. Process as claimed in claim 7, wherein it is Klebsiella pneumoniae.

9. Process as claimed in one of the claims 1 to 8, wherein the host organism is a eukaryotic or prokaryotic microorganism.

10. Process as claimed in claim 9, wherein it is E. coli.

11. Process as claimed in one of the claims 1 to 10, wherein the expression occurs under aerobic conditions.

12. Recombinant soluble protein with citrate lyase activity and a molecular weight of about 14,000 to 15,000 Dalton obtainable by a process as claimed in one of the claims 1 to 11.

13. Test kit for the determination of citric acid which comprises essentially the following components

(a) a protein with citrate lyase activity obtainable according to one of the claims 1 to 11,
(b) at least one protein with hydrogen-transferring activity
(c) nicotinamide adenine dinucleotide or a corresponding derivative in a reduced form and
(d) optionally suitable stabilizers, activators and/or substances to avoid or reduce interferences, and buffer solutions.

14. Test kit as claimed in claim 13, wherein L-malate dehydrogenase and optionally L-lactate dehydrogenase are used as the hydrogen-transferring enzymes.

15. Use of the enzyme obtainable according to claims 1 to 11 to determine citric acid.

Abstract

Process for the production of a protein with citrate lyase activity by expressing a suitable plasmid in a host organism and isolating the protein in an active form, wherein the plasmid contains the information from a gene cluster composed of at least six genes and an inducible promoter. Furthermore the invention concerns the use of the recombinant enzyme and a corresponding test kit for the determination of citric acid.

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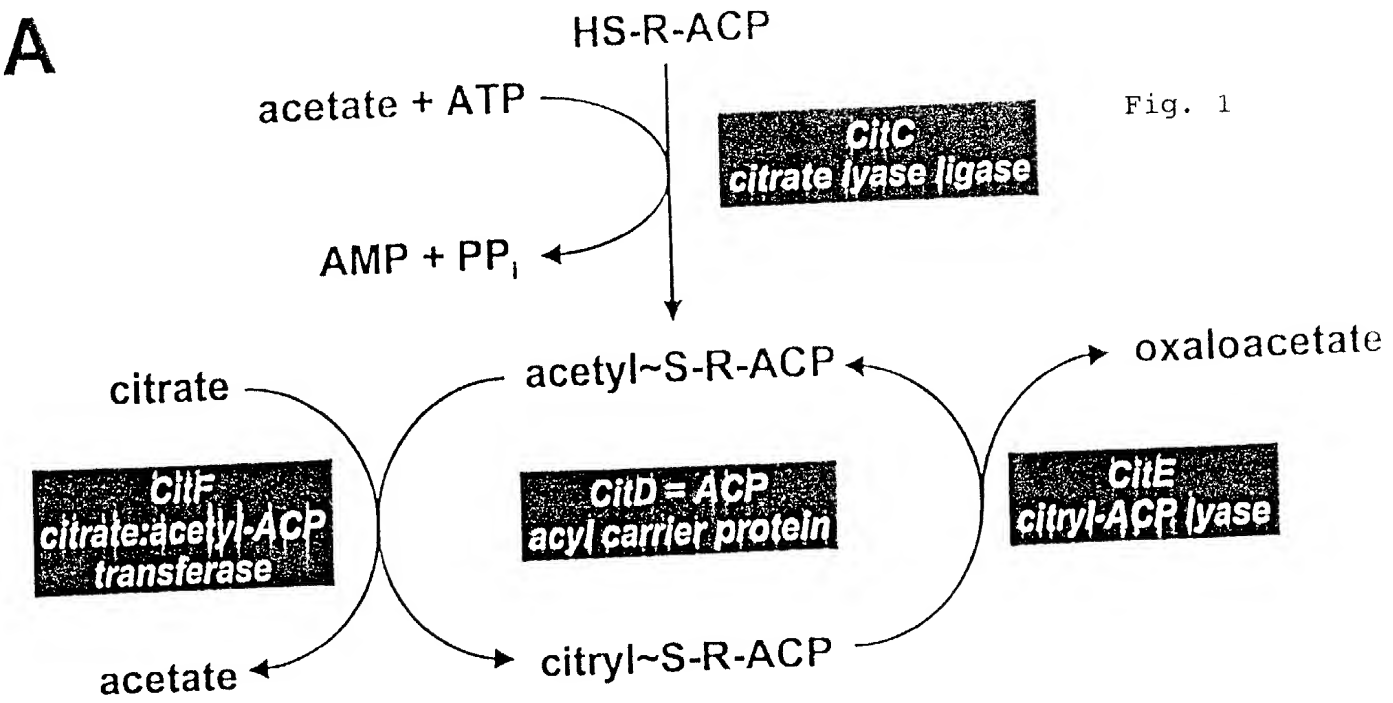

Fig. 1

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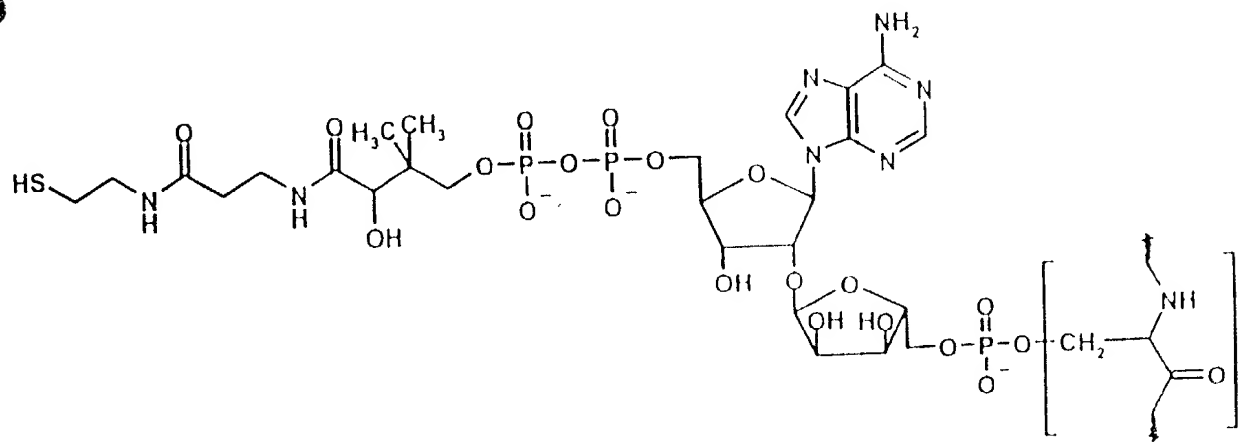

serine-14 residue of
the acyl carrier
protein

Fig. 2

1 kb

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Ligase Citrate lyase

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Ligase Citrate lyase

L.m.

Ligase Citrate lyase

Docket No.
BMID9975US

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
PROCESS FOR THE RECOMBINANT PRODUCTION OF HOLO-CITRATE LYASE

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application No. or PCT International Application Number _____

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
99119404.4	Europe	30 September 1999	☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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Fourth inventor's signature Date
Residence
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Full name of fifth inventor, if any
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Residence
Citizenship
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Full name of sixth inventor, if any
Sixth inventor's signature Date
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Citizenship
Post Office Address